

## II. REMARKS

Claims 2, 5-7, 17-19, 21-23 and 42 were examined and stand rejected in the outstanding Office Action. By this Amendment and Response, claims 2 and 42 have been amended in response to the obviousness rejection and to improve clarity of the claim language. Support for the amendments is found on page 6, lines 6 to 7 disclosing that yeast cells lack TNF- $\alpha$  and NF- $\kappa$ B signaling pathways and on page 14, lines 11 to 15 of the originally filed specification as well as Examples III and V.

Thus, the amendments to claims 2 and 42 do not raise an issue of new matter and entry thereof is respectfully requested. The amendments of the claims are not intended to be a dedication to the public of the subject matter of the claims as previously presented. Applicants reserve the right to pursue the subject matter of the claims as previously presented in a continuing application.

The amendments are made to place the application in condition for allowance or in better form for consideration on appeal. The amendments were not made earlier as it is Applicants' belief that the claims as previously presented were patentable over the cited art.

This amendment changes and deletes claims in this application. A detailed listing of all claims that are, or were, in the application, irrespective of whether the claims remain under examination in the application, is presented, with an appropriate defined status identifier.

After amending the claims as set forth above, claims 2, 5-7, 17-19, 21-23 and 42 are now pending in this application. In view of the remarks which follow, reconsideration and withdrawal of the objections and rejections are respectfully requested.

**35 U.S.C. § 103(a)**

Claims 2, 5-7, 17-19, 21-23 and 42 stand rejected under 35 U.S.C. § 103(a) as allegedly obvious over Rothwarf *et al.* in view of Traincard *et al.* and Epinat *et al.*

**1. NIK1 or MEKK1 phosphorylating IKK *in vitro* does not suggest that NIK1 or MEKK1 can phosphorylate IKK in yeast**

The Office alleged that Ling *et al.*, (1998) *Proc. Natl. Acad. Sci. USA* **95**:3792-9 teaches that NIK phosphorylates IKK *in vitro*, and Nakano *et al.*, (1998) *Proc. Natl. Acad. Sci. USA* **95**:3537-42 teaches that MEKK1 phosphorylates IKK *in vitro*. The Office further alleged that *in vitro* means "in the absence of any cellular context." Office Action mailed September 2, 2009, page 6, lines 1 to 2 (emphasis added). Based on this interpretation of the prior art, the Office then concluded that NIK or MEKK1 can phosphorylate IKK in yeast. Applicants respectfully disagree.

First, *in vitro* does not necessarily mean in the absence of any cellular context. Contrary, the term also refers to an experiment that occurs entirely in a cell or tissue outside the host organism, such as a human cell sustained in cell culture or in cell lysates sustained in a test tube. In fact, when Nakano *et al.* disclosed that MEKK1 phosphorylates IKK *in vitro*, the *in vitro* experiment supporting the finding was actually performed in human cell lysates. Applicants note that Nakano *et al.* cites and relies on Lee *et al.*, (1997) *Cell* 88:213-22 (attached as Exhibit A) for the conclusion that MEKK1 phosphorylates IKK *in vitro* (see page 3541, 2<sup>nd</sup> column, last paragraph). Applicants further note that in Lee *et al.*, the *in vitro* phosphorylation of I $\kappa$ B $\alpha$  kinase, an IKK protein, by MEKK1 was observed in **cytoplasmic** extracts of Hela cells, and **thus in the presence of cellular context**. See, e.g., page 216, 2<sup>nd</sup> column, first full paragraph.

Further, even though Ling *et al.* taught that NIK1 phosphorylates IKK outside the cells, both NIK1 and IKK were expressed and then isolated from 293 cells, a human cell line. See, e.g., page 3793, 1<sup>st</sup> column, first full paragraph and FIG. 1 legend, and page

3794, FIG. 3 legend. Ling *et al.*, further disclosed that NIK1 is activated before phosphorylating IKK. Page 3797, first full paragraph. Therefore, phosphorylation of IKK by NIK1 is highly likely due to activation of NIK1 in the human cells before it was isolated and incubated with IKK. Accordingly, the teachings of Ling *et al.*, do not suggest that a NIK1 protein expressed in yeast, which can not be activated in yeast due to the lack of the TNF- $\alpha$  and NF- $\kappa$ B signaling pathways in yeast, can activate IKK.

However, in a sincere effort to advance prosecution, claims 2 and 42 have been amended and is now directed to a method consisting essentially of the steps of transforming a yeast (claim 42) or a yeast that lacks TNF- $\alpha$  and NF- $\kappa$ B signaling pathways (claim 2) with one or more nucleotide sequences encoding an IKK subunit gamma ( $\gamma$ ) gene, an IKK subunit alpha ( $\alpha$ ) gene and an IKK subunit beta ( $\beta$ ) gene, growing said yeast (claims 2 and 42) and then separating IKK protein complex from said yeast (claim 2).

As noted in the Manual of Patent Examination and Procedure (MPEP) Section 2111.03 (Rev. 6, Sept. 2007) and the Federal Circuit's decision *PPG Indus. V. Guardian Indus. Corp.*, 156 F.3d 1351, 1354, *reh'g denied*, 1998 U.S. App. LEXIS 30846 (Fed. Cir. 1998), "consisting essentially of" signals that the invention excludes unlisted ingredients that materially affect the basic and novel properties of the invention. Therefore, claims 2 and 42 as amended excludes 1) yeast cells that, prior to the transformation, contain exogenously introduced, material components of the TNF- $\alpha$  and NF- $\kappa$ B signaling pathways, such as NIK and MEKK1, 2) additional steps of transforming the yeast with a sequence encoding a protein that materially affects the activities of the IKK protein complex, such as NIK and MEKK1, and 3) nucleotide sequence encoding a protein that materially affects the activities of the IKK protein complex, such as NIK and MEKK1, to be co-transformed to the yeast.

Claims 2 and 42 as amended, therefore, are restricted to the use of a transformed yeast in which proteins that can phosphorylate the IKK protein complex,

such as NIK and MEKK1, are not present, and thus the IKK protein complex is activated by autophosphorylation. Activation of the IKK protein complex by autophosphorylation, as disclosed in the specification and further explained below, is the unexpected finding of the current invention, and thus is not obvious in view of the prior art cited by the Office.

In view of the preceding amendments and remarks, consideration and withdrawal of this ground for rejection is respectfully requested.

**2. U.S. Patent No. 6,864,355 does not teach autophosphorylation of IKK that activates IKK**

The Office further argued that U.S. Patent No. 6,864,355 (“the ‘355 Patent”) taught that IKK- $\beta$  (1-733), which includes the C-terminal autophosphorylation site but lacks the NEMO binding site, had autophosphorylation and NF- $\kappa$ B activity, but the activity was not further enhanced by TNF- $\alpha$ . By contrast, the Office further alleged, that IKK- $\beta$  (1-744), which further includes the NEMO binding site, “had identical phosphorylation and NF- $\kappa$ B activity as wild type but the NF- $\kappa$ B activity and further autophosphorylation was further inducible by TNF $\alpha$ .” Office Action, page 7, lines 7 (emphasis added). Accordingly, the Office concluded that the ‘355 Patent taught that there is one or more autophosphorylation sites, other than the C-terminal autophosphorylation site, that are necessary for activation of the IKK protein complex. Applicants respectfully disagree.

First, Applicants note that, in the ‘355 Patent, phosphorylation of IKK occurred after the HeLa cells, a human transformed cell line, which expressed the exogenous IKK, was treated with TNF- $\alpha$ . Column 24, lines 46 to 49. The very fact that IKK required TNF- $\alpha$  pre-treatment in the human cell for the phosphorylation and activation indicates that it was appreciated by one of the skill in the art that introduction of IKK alone into the yeast, wherein the TNF- $\alpha$  signaling pathway does not exist, would not

lead to IKK phosphorylation or activation. The claimed invention, however, is directed to transforming a yeast with sequences encoding the IKK proteins, without the presence of material components of the TNF- $\alpha$  or NF- $\kappa$ B signaling pathways, and producing substantially homogenous, biologically functional and activated IKK protein complex.

Further, Applicants note that the '355 Patent discloses that TNF- $\alpha$  treatment enhanced IKK activity (see, column 25, lines 28 to 29) and led to rapid IKK autophosphorylation (see, column 25, lines 36 to 40). The '355 Patent, however, does not disclose whether this autophosphorylation contributed, in any way, to the enhanced activity of IKK, because the IKK protein might have well been activated and/or phosphorylated, or undergone structural or conformational changes, in the transformed HeLa cell before isolation.

Therefore, the '355 Patent does not teach that IKK proteins can be phosphorylated and activated without the presence of a human cellular context or the presence and activation of the TNF- $\alpha$  signaling pathway.

Because the prior art references cited by the Office do not teach that transforming IKK proteins alone into a yeast, without the presence of material components of the TNF- $\alpha$  and the NF- $\kappa$ B signaling pathways, results in phosphorylation and activation of the IKK protein complex, the claimed invention is not obvious over the references. Withdrawal of this ground for rejection, therefore, is respectfully requested.

### **III. CONCLUSION**

Applicants believe that the present application is now in condition for allowance. Favorable reconsideration of the application as amended is respectfully requested.

The Examiner is invited to contact the undersigned by telephone if it is felt that a telephone interview would advance the prosecution of the present application.

The Commissioner is hereby authorized to charge any additional fees which may be required regarding this application under 37 C.F.R. §§ 1.16-1.17, or credit any overpayment, to Deposit Account No. 19-0741. Should no proper payment be enclosed herewith, as by the credit card payment instructions in EFS-Web being incorrect or absent, resulting in a rejected or incorrect credit card transaction, the Commissioner is authorized to charge the unpaid amount to Deposit Account No. 19-0741. If any extensions of time are needed for timely acceptance of papers submitted herewith, Applicants hereby petition for such extension under 37 C.F.R. §1.136 and authorize payment of any such extensions fees to Deposit Account No. 19-0741.

Respectfully submitted,

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By 

FOLEY & LARDNER LLP  
Customer Number: 38706  
Telephone: (650) 251-1129  
Facsimile: (650) 856-3710

Antoinette F. Konski  
Attorney for Applicants  
Registration No. 34,202

Alex Y. Nie  
Attorney for Applicants  
Registration No. 60,523

# EXHIBIT A

# Activation of the I $\kappa$ B $\alpha$ Kinase Complex by MEKK1, a Kinase of the JNK Pathway

Frank S. Lee,\* Jeremiah Hagler,\* Zhijian J. Chen,<sup>†</sup> and Tom Maniatis\*

\*Department of Molecular and Cellular Biology  
Harvard University  
7 Divinity Avenue  
Cambridge, Massachusetts 02138

<sup>†</sup>ProScript, Inc.  
38 Sidney Street  
Cambridge, Massachusetts 02139

## Summary

Both NF- $\kappa$ B and c-Jun are activated by cytokines such as TNF- $\alpha$  and by stresses such as UV irradiation. A key step in the activation of NF- $\kappa$ B is the phosphorylation of its inhibitor, I $\kappa$ B $\alpha$ , by a ubiquitination-inducible multiprotein kinase complex (I $\kappa$ B $\alpha$  kinase). A central kinase in the c-Jun activation pathway is mitogen-activated protein kinase/ERK kinase kinase-1 (MEKK1). Here, we show that MEKK1 induces the site-specific phosphorylation of I $\kappa$ B $\alpha$  in vivo and, most strikingly, can directly activate the I $\kappa$ B $\alpha$  kinase complex in vitro. Thus, MEKK1 is a critical component of both the c-Jun and NF- $\kappa$ B stress response pathways. Since the I $\kappa$ B $\alpha$  kinase complex can be independently activated by ubiquitination or MEKK1-dependent phosphorylation, it may be an integrator of multiple signal transduction pathways leading to the activation of NF- $\kappa$ B.

## Introduction

Exposure of cells to certain cytokines or environmental stresses leads to the activation of the transcription factors NF- $\kappa$ B and c-Jun. In unstimulated cells, NF- $\kappa$ B is sequestered in the cytoplasm in a complex with I $\kappa$ B $\alpha$  or another member of the I $\kappa$ B family of inhibitor proteins (reviewed by Baldwin, 1996). Following stimulation by a variety of inducers, NF- $\kappa$ B is activated as a consequence of the phosphorylation and subsequent proteolytic degradation of the I $\kappa$ B protein. I $\kappa$ B $\alpha$  is phosphorylated at Serine residues 32 and 36 (Brockman et al., 1995; Brown et al., 1995; Traenckner et al., 1995), an event that targets this protein for degradation by the ubiquitin-proteasome pathway (Chen et al., 1995; reviewed by Baldwin, 1996). Recent studies have identified a large, multisubunit complex in HeLa cell cytoplasmic extracts that can phosphorylate I $\kappa$ B $\alpha$  at Ser-32 and -36 (the I $\kappa$ B $\alpha$  kinase, Chen et al., 1996). A novel property of this kinase, which was isolated from unstimulated cells, is that it can be activated in vitro by ubiquitination.

A second branch of the stress response is the c-Jun N-terminal kinase (JNK) pathway (also known as the stress-activated protein kinase pathway; for recent reviews and references see Karin, 1995, and Kyriakis and Avruch, 1996). The JNK cascade can be activated by small GTP-binding proteins that include Cdc42 and Rac1 (Coso et al., 1995; Minden et al., 1995), and protein

kinases that they directly activate, such as PAK (Bagrodia et al., 1995). MEKK1 (Lange-Carter et al., 1993) is then activated by these proteins by a mechanism yet to be determined. MEKK1 then activates MKK4, which in turn activates JNK (Sanchez et al., 1994; Yan et al., 1994; Derijard et al., 1995). Among the substrates of JNK are c-Jun, ATF-2, and Elk-1 (Derijard et al., 1994; Kyriakis et al., 1994; Gupta et al., 1995; Whitmarsh et al., 1995).

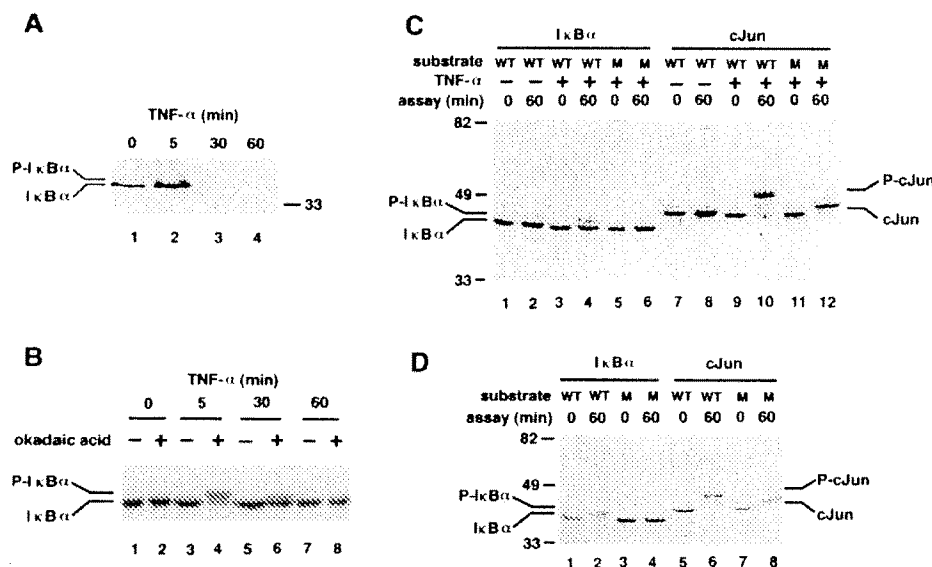
While the signal transduction cascade leading to the activation of JNK is relatively well defined, the steps leading to the phosphorylation of I $\kappa$ B $\alpha$  are poorly understood. Many of the stimuli that induce NF- $\kappa$ B, such as TNF- $\alpha$ , UV irradiation, and lipopolysaccharide, also activate the JNK cascade, thereby raising the possibility that the two pathways utilize common signal transduction components. Indeed, transfection of MEKK1 induces the degradation of I $\kappa$ B $\alpha$  and activates an NF- $\kappa$ B reporter gene (Hirano et al., 1996; Meyer et al., 1996), suggesting a link between the NF- $\kappa$ B and JNK pathways. These observations raise two critical questions. First, is MEKK1-induced degradation mediated by the same site-specific phosphorylation of I $\kappa$ B $\alpha$  identified in other contexts? Second, and more importantly, what is the target of MEKK1 in the NF- $\kappa$ B pathway? Here, we show that MEKK1 does in fact induce phosphorylation of I $\kappa$ B $\alpha$  at its sites of regulatory phosphorylation and that this occurs by direct activation of the I $\kappa$ B $\alpha$  kinase. Thus, MEKK1 is a critical coordinate regulator of both the NF- $\kappa$ B and JNK pathways.

## Results

### I $\kappa$ B $\alpha$ Kinase Activity Is Inducible by TNF- $\alpha$

Previous studies left open the question of whether the I $\kappa$ B $\alpha$  kinase is regulated by inducers of NF- $\kappa$ B. In those studies, the I $\kappa$ B $\alpha$  kinase was detected as an apparently constitutive activity in S100 cytoplasmic extracts prepared from uninduced HeLa cells using the hypotonic lysis procedure of Dignam et al., 1983 (Chen et al., 1996). Using an alternative method for preparing cytoplasmic extracts (a rapid lysis procedure detailed in Experimental Procedures), we now find that the I $\kappa$ B $\alpha$  kinase activity is inducible by TNF- $\alpha$ . HeLa cells were treated with TNF- $\alpha$  for differing lengths of time, and the rapid lysis extracts assayed for the presence of endogenous I $\kappa$ B $\alpha$  by Western blotting and for I $\kappa$ B $\alpha$  kinase activity by incubation with exogenous <sup>35</sup>S-labeled I $\kappa$ B $\alpha$  in the presence of okadaic acid (Figures 1A and 1B). We note that in these and all subsequent experiments okadaic acid is employed strictly as a phosphatase inhibitor (i.e., to preserve the phosphorylated I $\kappa$ B $\alpha$  species) rather than as an inducer of I $\kappa$ B $\alpha$  phosphorylation (Thevenin et al., 1990; Traenckner et al., 1995). Consistent with previous results (Henkel et al., 1993; Mellits et al., 1993), extracts from uninduced HeLa cells contain hypophosphorylated I $\kappa$ B $\alpha$  (Figure 1A, lane 1), but after only 5 min of TNF- $\alpha$  treatment a significant portion of the endogenous I $\kappa$ B $\alpha$  is phosphorylated (as revealed by the slower migrating I $\kappa$ B $\alpha$  species, lane 2). After 30 min of treatment, virtually





**Figure 1. I $\kappa$ B $\alpha$  Kinase Activity Is Inducible and Is Correlated With JNK Activity**

(A) HeLa cells were treated with TNF- $\alpha$  for the indicated times, and cytoplasmic extracts prepared by the rapid lysis procedure. Extracts (14  $\mu$ g) were then subjected to 10% SDS-PAGE, transferred to nitrocellulose membrane, and probed with anti-I $\kappa$ B $\alpha$  antibodies. The positions of unphosphorylated (I $\kappa$ B $\alpha$ ) and phosphorylated (P-I $\kappa$ B $\alpha$ ) I $\kappa$ B $\alpha$  are indicated to the left. Molecular weight marker (in kilodaltons) is indicated to the right.

(B) HeLa cells were treated with TNF- $\alpha$  for the indicated times, and cytoplasmic extracts prepared by the rapid lysis procedure. Extracts (9  $\mu$ g) were then incubated with  $^{35}$ S-labeled I $\kappa$ B $\alpha$  in the absence or presence of 6  $\mu$ M okadaic acid for 1 hr at 30°C. Reaction products were subjected to 9% SDS-PAGE and analyzed by autoradiography. The positions of unphosphorylated (I $\kappa$ B $\alpha$ ) and phosphorylated (P-I $\kappa$ B $\alpha$ ) I $\kappa$ B $\alpha$  are indicated to the left.

(C) HeLa cells were either mock or TNF- $\alpha$  (5 min) treated, and cytoplasmic extracts prepared by the rapid lysis procedure. Extracts (8  $\mu$ g) were then incubated with  $^{35}$ S-labeled wild-type (WT) or mutant (S32A/S36A, M) I $\kappa$ B $\alpha$ , or wild-type (WT) or mutant (S63A/S73A, M) c-Jun for 0 or 60 min at 30°C in the presence of 2.5  $\mu$ M okadaic acid. Reaction products were subjected to 10% SDS-PAGE and analyzed by autoradiography. Molecular weight markers (in kilodaltons) are indicated to the left. The positions of unphosphorylated (c-Jun) and phosphorylated (P-c-Jun) c-Jun are indicated to the right; those for I $\kappa$ B $\alpha$  are indicated to the left.

(D) HeLa cell S100 extracts (18  $\mu$ g) were incubated with  $^{35}$ S-labeled wild-type (WT) or mutant (S32A/S36A, M) I $\kappa$ B $\alpha$ , or wild-type (WT) or mutant (S63A/S73A, M) c-Jun for 0 or 60 min at 30°C in the presence of 2.5  $\mu$ M okadaic acid. Reaction products were subjected to 10% SDS-PAGE and analyzed by autoradiography. Molecular weight markers (in kilodaltons) are indicated to the left.

all of the I $\kappa$ B $\alpha$  is degraded (lane 3). Parallel assays with the same extracts reveal that the I $\kappa$ B $\alpha$  kinase activity is absent in uninduced cells (Figure 1B, lane 2) (activity is weakly detectable when higher concentrations of these extracts are employed). However, after exposure of cells to TNF- $\alpha$  for only 5 min, I $\kappa$ B $\alpha$  kinase activity can be readily detected, as evidenced by the slower-migrating I $\kappa$ B $\alpha$  species (lane 4). Fractionation of these extracts by gel filtration reveals that the TNF- $\alpha$ -inducible I $\kappa$ B $\alpha$  kinase activity resides in a large (approximately 700 kDa) complex (data not shown). Interestingly, this activity persists and is present after 30 min of TNF- $\alpha$  induction (lane 6), a time at which the endogenous I $\kappa$ B $\alpha$  has been degraded (Figure 1A, lane 3). I $\kappa$ B $\alpha$  kinase activity is essentially absent at 60 min (Figure 1B, lane 8). It is formally possible that the I $\kappa$ B $\alpha$  kinase activity is constitutive and that TNF- $\alpha$  treatment simply results in the inactivation of a phosphatase in the extract that dephosphorylates I $\kappa$ B $\alpha$ . To address this possibility, the same extracts were incubated with  $^{35}$ S-labeled I $\kappa$ B $\alpha$  in the absence of okadaic acid (Figure 1B, lanes 1, 3, 5, and 7). Under these conditions, the I $\kappa$ B $\alpha$  mobility shift is completely abolished (for example, compare lanes 3 and 4). Thus, the effects of TNF- $\alpha$  treatment cannot be accounted for

solely by inactivation of an okadaic acid-sensitive I $\kappa$ B $\alpha$  phosphatase, implying that TNF- $\alpha$  treatment induces I $\kappa$ B $\alpha$  kinase activity. Furthermore, the rapid induction of I $\kappa$ B $\alpha$  kinase activity correlates with the rapid appearance of the phosphorylated form of I $\kappa$ B $\alpha$ .

#### Coordinate Activation of I $\kappa$ B $\alpha$ Kinase and JNK Activities In Vitro

TNF- $\alpha$  treatment also leads to the activation of c-Jun by JNK (Hibi et al., 1993). We therefore carried out experiments to determine whether the I $\kappa$ B $\alpha$  kinase and JNK are coactivated in extracts from TNF- $\alpha$ -treated cells. Cytoplasmic extracts from uninduced and TNF- $\alpha$ -induced HeLa cells were incubated with in vitro translated,  $^{35}$ S-labeled I $\kappa$ B $\alpha$  or c-Jun, and the proteins fractionated by SDS-PAGE (Figure 1C). As before, I $\kappa$ B $\alpha$  kinase activity is detected in extracts from TNF- $\alpha$ -induced cells but not in those from uninduced cells (compare lanes 2 and 4). The specificity of phosphorylation is indicated by the fact that the S32A/S36A mutation in I $\kappa$ B $\alpha$  completely abolishes the I $\kappa$ B $\alpha$  shift (lane 6). Similarly, extracts from TNF- $\alpha$ -induced cells show JNK activity, as evidenced by the appearance of a c-Jun species with markedly reduced mobility (compare lanes

9 and 10). The observed shift is a result of JNK activity, since amino acid substitutions at the sites of JNK phosphorylation (S63A/S73A) in c-Jun abolish this shift (lane 12). We note that a distinct shift is observed with the c-Jun mutant, suggesting that JNK may phosphorylate c-Jun at residues other than Ser-63 and -73. Importantly, extracts from uninduced cells show no significant JNK activity (lane 8). Thus, both the I $\kappa$ B $\alpha$  kinase and JNK activities are activated in the rapid lysis extracts prepared from TNF- $\alpha$ -treated, but not untreated, cells.

By contrast, the I $\kappa$ B $\alpha$  kinase activity is readily detected in S100 cytoplasmic extracts prepared from unstimulated HeLa cells using the hypotonic lysis procedure (Chen et al., 1995). It is possible that stress pathways are activated by this procedure, since another form of osmotic stress, hyperosmolar shock, has been shown to be an efficient activator of the JNK pathway (Galcheva-Gargova et al., 1994). Indeed, both the I $\kappa$ B $\alpha$  kinase and the JNK activities were detected when the S100 extracts were incubated for 60 min (Figure 1D). A time-dependent activation of JNK was detected when the S100 extracts were incubated and then examined by an in-gel kinase assay employing the JNK substrate ATF-2 (data not shown). Thus, both the JNK and I $\kappa$ B $\alpha$  kinase may be activated during incubation of the S100 extracts, possibly owing to the hypotonic lysis conditions.

#### MEKK1 Activates NF- $\kappa$ B In Vivo

Transient transfection studies were conducted to examine the relationship between the activation of the I $\kappa$ B $\alpha$  kinase and JNK in vivo. The IFN- $\beta$  enhancer contains multiple positive regulatory domains (PRDs) that bind distinct transcription factors, including NF- $\kappa$ B (PRDII) and ATF-2/c-Jun (PRDIV) (reviewed in Thanos et al., 1993). HeLa cells were transfected with reporters linked to either two copies of PRDII (PII), six copies of PRDIV (PIV), or the intact IFN- $\beta$  enhancer (IFN), which includes these as well as other PRDs, and either an expression vector for MEKK1 or an expression vector alone. Note that in these and all subsequent experiments, MEKK1 and MEKK1 $\Delta$  refer to the 672 and 321 residue C-terminal fragments, respectively, of the full-length molecule (for

discussion, see Xu et al., 1996). Both kinases are constitutively active and indistinguishable in transfection studies. As expected, MEKK1 activates the reporter linked to a multimer of PRDIV (Figure 2A), which binds to either an ATF-2 homodimer or an ATF-2/c-Jun heterodimer (Du et al., 1993). Both ATF-2 and c-Jun contain transcriptional activation domains that are phosphorylated by the JNK pathway (Gupta et al., 1995). Importantly, MEKK1 also activates the PRDII reporter. MEKK1 does not activate all promoters, since its effect on a reporter gene containing the intact IFN- $\beta$  enhancer is only marginal. This enhancer contains additional PRDs that bind factors other than NF- $\kappa$ B or ATF-2/c-Jun (see Thanos et al., 1993). As expected, the IFN- $\beta$  enhancer is effectively activated by virus infection, which activates all of the PRDs. We conclude that MEKK1 can activate both ATF-2/c-Jun and NF- $\kappa$ B in vivo.

To examine whether MEKK1 plays a role in the activation of NF- $\kappa$ B in response to TNF- $\alpha$ , HeLa cells were transfected with a PRDII reporter and expression vector for catalytically inactive (K432M) MEKK1 $\Delta$ , or empty expression vector. Some cells were then stimulated with TNF- $\alpha$ , and subsequently all cells were harvested and examined for reporter gene activity. As expected, TNF- $\alpha$  activates the PRDII reporter efficiently (Figure 2B). By contrast, the mutant MEKK1 $\Delta$  (K432M) inhibits both the basal and TNF- $\alpha$ -induced activity of this reporter, thus behaving as a dominant negative inhibitor, as has also been shown by Hirano et al. (1996). Similar results are observed in L929 cells (Figure 2C). As a negative control, cAMP activation of a cAMP response element reporter is not significantly affected by dominant negative MEKK1 $\Delta$  (Figure 2D). These results suggest that MEKK1 plays a role in TNF- $\alpha$  activation of NF- $\kappa$ B.

#### MEKK1 Activation of NF- $\kappa$ B Occurs through Site-Specific Phosphorylation of I $\kappa$ B $\alpha$

Numerous stimuli that activate NF- $\kappa$ B have been shown to induce site-specific phosphorylation of I $\kappa$ B $\alpha$  at Ser-32 and -36 (Brockman et al., 1995; Brown et al., 1995; Traenckner et al., 1995). Experiments were therefore conducted to examine whether MEKK1 induces this same phosphorylation. HeLa cells were transfected with

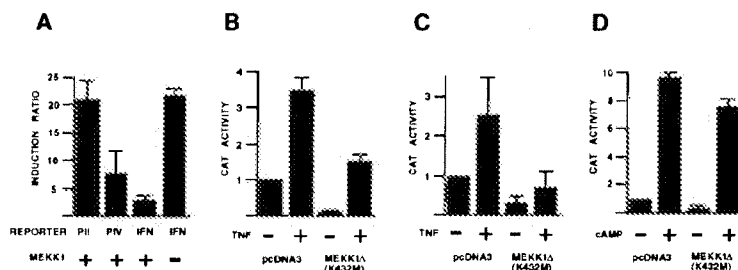
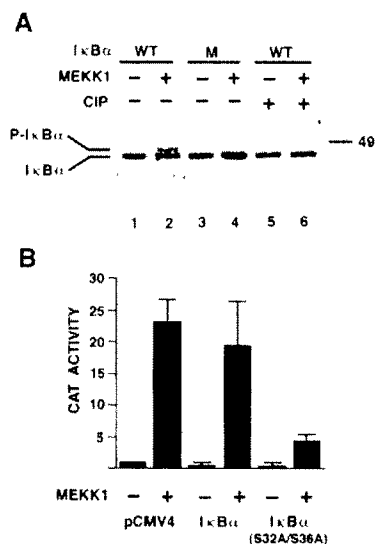


Figure 2. MEKK1 Activates NF- $\kappa$ B In Vivo

(A) HeLa cells were transfected with 3  $\mu$ g of (PRDII)<sub>2</sub>CAT, 2  $\mu$ g pCMV-lacZ, and 4  $\mu$ g of pCMV5-MEKK1 or pCDNA3. Twenty-six to 28 hr posttransfection, cells in one well were infected with Sendai virus for 15 hr. All cells were harvested 41 to 43 hr posttransfection. CAT activities were normalized to protein concentrations of cell extracts. Shown are the averages and standard deviations from three independent experiments.

(B and C) HeLa (B) or L929 (C) cells were transfected with 3  $\mu$ g of (PRDII)<sub>2</sub>CAT, 2  $\mu$ g pCMV-lacZ, and 4  $\mu$ g of pCDNA3-FlagMEKK1 $\Delta$  (K432M) or pCDNA3. Forty to 41 hr posttransfection, some cells were treated with 20 ng/ml mouse TNF- $\alpha$  (Boehringer) for 8 hr. All cells were harvested 48 to 49 hr posttransfection. CAT activities were normalized to those for  $\beta$ -galactosidase. Shown are the averages and standard deviations from (B) one experiment performed in triplicate or (C) three independent experiments.

(D) L929 cells were transfected with 3  $\mu$ g of (CRE)<sub>3</sub>CAT, 2  $\mu$ g pCMV-lacZ, and 4  $\mu$ g of pCDNA3-FlagMEKK1 $\Delta$  (K432M) or pCDNA3. Forty to 41 hr posttransfection, some cells were treated with 1 mM 8-Br-cAMP for 8 hr. All cells were harvested 48 to 49 hr posttransfection. CAT activities were normalized to those for  $\beta$ -galactosidase. Shown are the averages and standard deviations from three independent experiments.



**Figure 3. MEKK1 Activation of NF- $\kappa$ B Is through Site-Specific Phosphorylation of I $\kappa$ B $\alpha$**

(A) HeLa cells were transfected with 0.3  $\mu$ g of expression vectors for wild-type (WT) (pCMV4-FlagI $\kappa$ B $\alpha$ ) or mutant (M) (pCMV4-FlagI $\kappa$ B $\alpha$  [S32A/S36A]) I $\kappa$ B $\alpha$ , 3  $\mu$ g of pCMV5-MEKK1 or pCMV5, and 3  $\mu$ g of SP72. Forty-one hr posttransfection, epitope-tagged I $\kappa$ B $\alpha$  was immunoprecipitated, and some samples were treated with calf intestinal alkaline phosphatase (CIP). All samples were then subjected to 10% SDS-PAGE, transferred to nitrocellulose membrane, and probed with anti-I $\kappa$ B $\alpha$  antibodies.

(B) HeLa cells were transfected with 3  $\mu$ g of (PRDII)<sub>2</sub>CAT, 10 ng of pCMV4-FlagI $\kappa$ B $\alpha$ , pCMV4-FlagI $\kappa$ B $\alpha$  (S32A/S36A) or pCMV4, and 4  $\mu$ g of pCMV5-MEKK1 or pCMV5. Cells were harvested 38 to 43 hr posttransfection. CAT activities were normalized to protein concentrations of cell extracts. Shown are the averages and standard deviations from three independent experiments.

expression vectors for Flag-tagged wild-type or mutant (S32A/S36A) I $\kappa$ B $\alpha$ , and an expression vector for MEKK1 or the expression vector alone. I $\kappa$ B $\alpha$  was then immunoprecipitated with anti-Flag antibodies, and then visualized by Western blotting using anti-I $\kappa$ B $\alpha$  antibodies. MEKK1 induces the appearance of an I $\kappa$ B $\alpha$  species with reduced mobility compared to that isolated from uninduced cells (Figure 3A, lane 2). This species is sensitive to treatment with calf intestinal alkaline phosphatase (compare lanes 2 and 6), consistent with its being a phosphorylated form of I $\kappa$ B $\alpha$ . Most importantly, Ser-to-Ala mutations at residues 32 and 36 of I $\kappa$ B $\alpha$  abolish this species (lane 4). Thus, MEKK1 induces site-specific phosphorylation of I $\kappa$ B $\alpha$  at Ser-32 and -36.

If the MEKK1-dependent phosphorylation of these serine residues is functionally important, their substitution by alanines should make I $\kappa$ B $\alpha$  degradation and hence NF- $\kappa$ B activation refractory to MEKK1 stimulation. To test this possibility, HeLa cells were transfected with the PRDII reporter gene and expression vectors for wild-type or mutant (S32A/S36A) I $\kappa$ B $\alpha$  or expression vector alone and either expression vector for MEKK1 or expression vector alone. HeLa cells transfected with wild-type I $\kappa$ B $\alpha$  display MEKK1-inducible reporter gene activity that is virtually the same as that of control (Figure 3B). In contrast, HeLa cells transfected with mutant I $\kappa$ B $\alpha$

display MEKK1-inducible activity that is significantly diminished. Other experiments indicate that it is necessary to transfect approximately 10-fold higher amounts of wild-type I $\kappa$ B $\alpha$  plasmid in order to observe a comparably reduced MEKK1-inducible reporter gene activity (data not shown). These experiments therefore indicate that site-specific phosphorylation of I $\kappa$ B $\alpha$  at Ser-32 and -36 plays a critical role in the MEKK1-dependent activation of NF- $\kappa$ B.

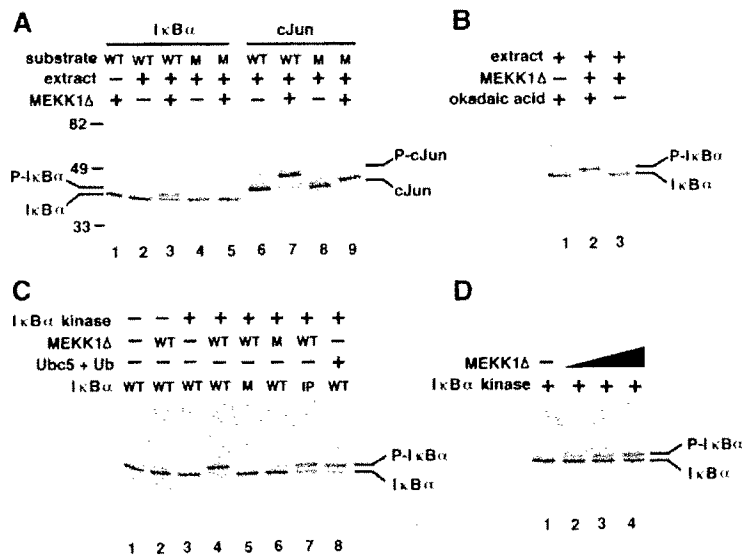
### MEKK1 Coordinately Activates the I $\kappa$ B $\alpha$ Kinase and JNK In Vitro

The transfection data show that MEKK1 expression leads to the site-specific phosphorylation of I $\kappa$ B $\alpha$ . To investigate the possibility that MEKK1 activates the I $\kappa$ B $\alpha$  kinase, cytoplasmic extracts were prepared from uninduced HeLa cells by the rapid lysis procedure and then treated with recombinant MEKK1 $\Delta$ . In the absence of MEKK1 $\Delta$ , these extracts show no significant site-specific I $\kappa$ B $\alpha$  kinase or JNK activity when incubated with in vitro-translated, <sup>35</sup>S-labeled I $\kappa$ B $\alpha$  or c-Jun, respectively (Figure 4A, lanes 2 and 6). By contrast, when recombinant MEKK1 $\Delta$  was added to the extract, site-specific phosphorylation of c-Jun was observed (compare lanes 7 and 9). Importantly, site-specific I $\kappa$ B $\alpha$  kinase activity was also observed (compare lanes 3 and 5), but MEKK1 $\Delta$  alone fails to induce this site-specific phosphorylation (lane 1). To rule out the possibility that MEKK1 $\Delta$  inactivates an I $\kappa$ B $\alpha$  phosphatase, extracts were incubated with MEKK1 $\Delta$  in the absence or presence of the phosphatase inhibitor okadaic acid (Figure 4B). In the absence of okadaic acid, the MEKK1 $\Delta$ -induced I $\kappa$ B $\alpha$  shift is largely abolished (compare lanes 2 and 3). Thus, the effects of MEKK1 $\Delta$  cannot be accounted for solely by the inactivation of an okadaic acid-sensitive I $\kappa$ B $\alpha$  phosphatase, implying that MEKK1 $\Delta$  activates the I $\kappa$ B $\alpha$  kinase. We conclude that MEKK1 $\Delta$  coordinately activates the I $\kappa$ B $\alpha$  kinase and JNK pathways in cytoplasmic extracts.

### MEKK1 Directly Activates the I $\kappa$ B $\alpha$ Kinase

In the JNK pathway, MEKK1 phosphorylates and activates MKK4, which, in turn, activates JNK. It is therefore possible that I $\kappa$ B $\alpha$  could be a substrate for MEKK1, MKK4, or JNK. When expressed as recombinant proteins, however, neither MKK4 nor JNK1 phosphorylated I $\kappa$ B $\alpha$ , with appropriate control experiments demonstrating that these proteins were enzymatically active (data not shown). MEKK1 $\Delta$  did phosphorylate I $\kappa$ B $\alpha$  directly; however, the degree of phosphorylation was over 10-fold less than that seen with MKK4 as a substrate, and, as shown below, MEKK1 $\Delta$  does not phosphorylate I $\kappa$ B $\alpha$  at Ser-32 or -36. In addition, recent experiments indicate the I $\kappa$ B $\alpha$  kinase activity resides in a large, approximately 700 kDa, multiprotein complex (Chen et al., 1996), and Western blotting of this complex fails to reveal the presence of MEKK1, MKK4, JNK1, or JNK2 (data not shown). A reasonable hypothesis, therefore, is that MEKK1 or one of the downstream kinases phosphorylates I $\kappa$ B $\alpha$  indirectly by stimulating the I $\kappa$ B $\alpha$  kinase.

To distinguish between these possibilities, MEKK1 $\Delta$  was incubated with purified, ubiquitination-inducible



**Figure 4. MEKK1 Directly Activates the I $\kappa$ B $\alpha$  Kinase**

(A) Uninduced HeLa cell cytoplasmic extracts (2  $\mu$ g) prepared by the rapid lysis procedure were incubated with  $^{35}$ S-labeled wild-type (WT) or mutant (S32A/S36A, M) I $\kappa$ B $\alpha$ , or wild-type (WT) or mutant (S63A/S73A, M) c-Jun in the absence or presence of 20 ng MEKK1 $\Delta$  for 1 hr at 30°C in the presence of 2.5  $\mu$ M okadaic acid. An additional incubation (lane 1) contained 20 ng MEKK1 $\Delta$  and  $^{35}$ S-labeled I $\kappa$ B $\alpha$  in the absence of extract. Reaction products were subjected to 10% SDS-PAGE and analyzed by autoradiography. Molecular weight markers (in kilodaltons) are indicated to the left.

(B) Uninduced HeLa cell cytoplasmic extracts (2  $\mu$ g) prepared by the rapid lysis procedure were incubated with  $^{35}$ S-labeled I $\kappa$ B $\alpha$  in the absence or presence of 20 ng MEKK1 $\Delta$  and/or 2.5  $\mu$ M okadaic acid for 1 hr at 30°C. Reaction products were subjected to 10% SDS-PAGE and analyzed by autoradiography.

(C) Purified I $\kappa$ B $\alpha$  kinase was incubated with wild-type (WT), mutant (S32A/S36A) (M),

or immunoprecipitated wild-type (IP)  $^{35}$ S-labeled FlagI $\kappa$ B $\alpha$  in the absence or presence of 20 ng wild-type (WT) or mutant (K432M) (M) MEKK1 $\Delta$ , or 0.9  $\mu$ g GST-Ubc5 + 0.5 mg/ml ubiquitin for 1 hr at 30°C in the presence of 2.5  $\mu$ M okadaic acid. An additional incubation (lane 1) contained 20 ng MEKK1 $\Delta$  and  $^{35}$ S-labeled FlagI $\kappa$ B $\alpha$  in the absence of I $\kappa$ B $\alpha$  kinase. Reaction products were subjected to 10% SDS-PAGE and analyzed by autoradiography. In lane 8, additional bands at higher molecular weights than phosphorylated I $\kappa$ B $\alpha$  represent ubiquitinated I $\kappa$ B $\alpha$  species, owing to the presence of ubiquitination components (Chen et al., 1995, 1996).

(D) Purified I $\kappa$ B $\alpha$  kinase in the absence or presence of 5, 10, or 20 ng MEKK1 $\Delta$  was incubated with  $^{35}$ S-labeled FlagI $\kappa$ B $\alpha$  for 1 hr at 30°C in the presence of 2.5  $\mu$ M okadaic acid. Reaction products were subjected to 10% SDS-PAGE and analyzed by autoradiography.

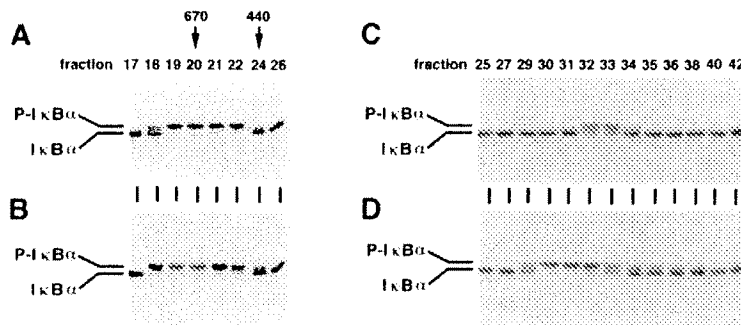
I $\kappa$ B $\alpha$  kinase (Chen et al., 1996) and in vitro-translated,  $^{35}$ S-labeled I $\kappa$ B $\alpha$  (Figure 4C). In the absence of the ubiquitin-conjugating enzyme Ubc5 and ubiquitin, the I $\kappa$ B $\alpha$  kinase is inactive (lane 3), while in their presence the kinase is active, as evidenced by the shift in mobility of the  $^{35}$ S-labeled I $\kappa$ B $\alpha$  (lane 8), as shown previously (Chen et al., 1996). Strikingly, addition of MEKK1 $\Delta$  independently activates the I $\kappa$ B $\alpha$  kinase (lane 4), while MEKK1 $\Delta$  alone does not site-specifically phosphorylate I $\kappa$ B $\alpha$  (lane 2). That this shift reflects phosphorylation of I $\kappa$ B $\alpha$  at Ser-32 and -36 is indicated by the fact that the S32A/S36A mutant fails to display this shift (lane 5). The activation of the I $\kappa$ B $\alpha$  kinase depends on the catalytic activity of MEKK1 $\Delta$ , since mutant MEKK1 $\Delta$ (K432M) fails to activate (lane 6). Neither recombinant MKK4 nor JNK1 augments MEKK1 $\Delta$  stimulation of the I $\kappa$ B $\alpha$  kinase (data not shown), ruling out the possibility that MEKK1 $\Delta$  activation of the I $\kappa$ B $\alpha$  kinase is mediated by an insect (Sf9) cell MKK4- or JNK-like activity copurifying in trace amounts with the MEKK1 $\Delta$  protein. To eliminate the possibility that MEKK1 $\Delta$  activation of the I $\kappa$ B $\alpha$  kinase is mediated through a factor present in the wheat germ extract employed for in vitro translation of I $\kappa$ B $\alpha$ , immunoprecipitated I $\kappa$ B $\alpha$  was employed as a substrate. As shown in lane 7, this I $\kappa$ B $\alpha$  is also a substrate for MEKK1 $\Delta$ -activated I $\kappa$ B $\alpha$  kinase. We conclude that MEKK1 $\Delta$  activation of the I $\kappa$ B $\alpha$  kinase is direct. Additional experiments indicate that MEKK1 $\Delta$  is a potent activator of the I $\kappa$ B $\alpha$  kinase (Figure 4D), with activation demonstrable with MEKK1 $\Delta$  doses as low as 5 ng (lane 2). Finally, I $\kappa$ B $\alpha$  complexed with RelA (p65) is a substrate for MEKK1 $\Delta$ -activated I $\kappa$ B $\alpha$  kinase, just as it is for the ubiquitination-activated kinase (data not shown).

### The MEKK1-Inducible I $\kappa$ B $\alpha$ Kinase Is a High Molecular Weight Complex

To further examine the relationship between the MEKK1 $\Delta$ - and the ubiquitination-inducible I $\kappa$ B $\alpha$  kinase previously reported (Chen et al., 1996), we fractionated HeLa cell cytoplasmic extracts and assayed for both activities (Figure 5). Notably, both MEKK1 $\Delta$ - and ubiquitination-inducible I $\kappa$ B $\alpha$  kinase activities copurify during the first four steps of fractionation, which include ion exchange chromatography, ammonium sulfate fractionation, hydroxylapatite chromatography (data not shown), and gel filtration (Figures 5A and 5B). With regard to the gel filtration step, the peak of MEKK1 $\Delta$ -inducible I $\kappa$ B $\alpha$  kinase activity elutes at a position (fractions 19 to 20) corresponding to a native molecular weight of approximately 700 kDa, indistinguishable from that of the ubiquitination-inducible I $\kappa$ B $\alpha$  kinase (Chen et al., 1996). Further fractionation by anion exchange chromatography reveals that the MEKK1 $\Delta$ -inducible I $\kappa$ B $\alpha$  kinase activity elutes in a broader peak than the ubiquitination-inducible activity (Figures 5C and 5D). Thus, some fractions (e.g., 32 and 33) are inducible by both MEKK1 $\Delta$  and ubiquitination, while others (e.g., 29 and 30) are inducible only by MEKK1 $\Delta$ . We conclude that the two kinase complexes are largely similar but may have subtle differences in structure or subunit composition.

### MEKK1 Is a Selective Activator of the I $\kappa$ B $\alpha$ Kinase

To examine the specificity of MEKK1 $\Delta$  activation of the I $\kappa$ B $\alpha$  kinase, three additional kinases, casein kinase II (CKII), protein kinase A (PKA), and protein kinase C $\zeta$  (PKC $\zeta$ ), were assayed for their capacity to activate the



**Figure 5. The MEKK1-Inducible  $\text{I}\kappa\text{B}\alpha$  Kinase Is a High Molecular Weight Complex**

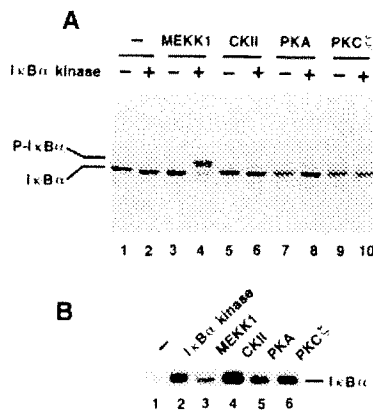
HeLa cell cytoplasmic extracts were fractionated as described in Experimental Procedures and then chromatographed on (A and B) a Superdex-200 gel filtration column followed by a (C and D) Mono-Q ion exchange column. Fractions were assayed for  $\text{I}\kappa\text{B}\alpha$  kinase activity with  $^{35}\text{S}$ -labeled Flag $\text{I}\kappa\text{B}\alpha$  in the presence of either (A and C) ubiquitination components or (B and D) 10 ng MEKK1 $\Delta$  for 1 hr at 37°C in the presence of 3  $\mu\text{M}$  okadaic acid. Reaction products were subjected to 9% SDS-PAGE and analyzed by autoradiography. The numbers 670 and 440 in (A) and (B) indicate elution positions of molecular weight standards (in kilodaltons).

$\text{I}\kappa\text{B}\alpha$  kinase (Figure 6). In marked contrast to MEKK1 $\Delta$ , none of these enzymes activates the  $\text{I}\kappa\text{B}\alpha$  kinase (Figure 6A). The enzymatic activity of the kinases is demonstrated by their roughly comparable degree of phosphorylation of recombinant  $\text{I}\kappa\text{B}\alpha$  with  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  (Figure 6B). The experiment shown in Figure 6A also demonstrates that none of the enzymes, aside from the  $\text{I}\kappa\text{B}\alpha$  kinase, phosphorylates  $\text{I}\kappa\text{B}\alpha$  at Ser-32 or -36 under the conditions employed. Phosphorylation by these other enzymes presumably occurs at residues other than Ser-32 or -36.

#### MEKK1 Activates the $\text{I}\kappa\text{B}\alpha$ Kinase Complex by Phosphorylation

The fact that the catalytically inactive MEKK1 $\Delta$  does not activate the  $\text{I}\kappa\text{B}\alpha$  kinase (Figure 4C, lane 6) strongly

suggests that MEKK1 $\Delta$  phosphorylates the  $\text{I}\kappa\text{B}\alpha$  kinase complex. To further examine this possibility, MEKK1 $\Delta$ -activated  $\text{I}\kappa\text{B}\alpha$  kinase was incubated with or without calf intestinal alkaline phosphatase, and the  $\text{I}\kappa\text{B}\alpha$  kinase was then assayed for activity against  $^{35}\text{S}$ -labeled  $\text{I}\kappa\text{B}\alpha$  in the absence or presence of MEKK1 $\Delta$ . As shown in Figure 7A, treatment of the MEKK1 $\Delta$ -activated  $\text{I}\kappa\text{B}\alpha$  kinase with phosphatase results in inactivation of  $\text{I}\kappa\text{B}\alpha$  kinase activity (compare lanes 2 and 4). Subsequent addition of MEKK1 $\Delta$  results in substantial, though incomplete, restoration of  $\text{I}\kappa\text{B}\alpha$  kinase activity (compare lanes 2, 3, and 4). To extend these observations, the purified  $\text{I}\kappa\text{B}\alpha$  kinase was incubated with or without MEKK1 $\Delta$  in the presence of  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  (Figure 7B). In the absence of MEKK1 $\Delta$ ,  $^{32}\text{P}$  label was incorporated into three subunits (approximately 200, 180, and 120 kDa) of the  $\text{I}\kappa\text{B}\alpha$  kinase complex (lane 2). In the presence of MEKK1 $\Delta$ ,  $^{32}\text{P}$  label was incorporated into three additional subunits of molecular weights of approximately 105, 64, and 54 kDa (lane 3). In conjunction with the experiment employing the catalytically inactive MEKK1 $\Delta$  (Figure 4C), these experiments show that MEKK1 $\Delta$  activates the  $\text{I}\kappa\text{B}\alpha$  kinase complex by phosphorylation. We are currently investigating which subunit(s) is the substrate(s) for MEKK1.



**Figure 6. MEKK1 Is a Selective Activator of the  $\text{I}\kappa\text{B}\alpha$  Kinase**

(A) MEKK1 $\Delta$  (10 ng), CKII (0.35 ng, 250 mU, New England Biolabs), PKA (0.8 ng, 1 mU, New England Biolabs), and PKC $\zeta$  (15 ng, Pan-Vera), either alone or in combination with purified  $\text{I}\kappa\text{B}\alpha$  kinase, were incubated with  $^{35}\text{S}$ -labeled Flag $\text{I}\kappa\text{B}\alpha$  for 30 min at 30°C in the presence of 2.5  $\mu\text{M}$  okadaic acid. An additional incubation (lane 2) contained purified  $\text{I}\kappa\text{B}\alpha$  kinase and  $^{35}\text{S}$ -labeled Flag $\text{I}\kappa\text{B}\alpha$ . Reaction products were subjected to 10% SDS-PAGE and analyzed by autoradiography.

(B) Purified  $\text{I}\kappa\text{B}\alpha$  kinase, MEKK1 $\Delta$ , CKII, PKA, and PKC $\zeta$  in the amounts employed in (A) were incubated with 0.5  $\mu\text{g}$  (His) $_6$  $\text{I}\kappa\text{B}\alpha$  in the presence of  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ . Reaction products were subjected to 10% SDS-PAGE and analyzed by autoradiography. Relative kinase activities determined by phosphorimager analysis for the  $\text{I}\kappa\text{B}\alpha$  kinase, MEKK1 $\Delta$ , CKII, PKA, and PKC $\zeta$  are 1, 0.6, 2.2, 1.0, and 1.3, respectively.

#### Discussion

The activation of the  $\text{I}\kappa\text{B}\alpha$  kinase and JNK pathway by a single protein, MEKK1, provides a compelling explanation for how multiple stimuli can simultaneously activate these two distinct kinases (Figure 8). Thus, TNF- $\alpha$ , UV irradiation, and lipopolysaccharide have all been shown to activate the JNK pathway. Their implied activation of MEKK1 now provides a mechanism for the activation of the  $\text{I}\kappa\text{B}\alpha$  kinase. Stimuli such as phorbol myristate acetate/ionomycin could also potentially act through this pathway; in T cells, for example, phorbol myristate acetate and ionomycin synergistically activate the JNK pathway (Su et al., 1994) and thus may activate the  $\text{I}\kappa\text{B}\alpha$  kinase through MEKK1. The coordinate activation of the  $\text{I}\kappa\text{B}\alpha$  kinase and JNK raises the possibility that potential upstream activators of MEKK1, such as the small GTP-binding proteins Rac1, Cdc42, and Ras, as well as protein kinases that they activate, such as PAK, may also be common elements of a single upstream signal transduction mechanism.

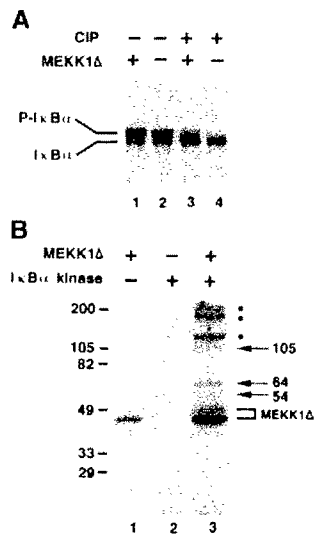


Figure 7. MEKK1 Activates the I $\kappa$ B $\alpha$  Kinase Complex by Phosphorylation

(A) MEKK1 $\Delta$ -activated I $\kappa$ B $\alpha$  kinase was incubated with or without calf intestinal alkaline phosphatase (CIP, as indicated), and subsequently incubated with or without 12 ng MEKK1 $\Delta$  (as indicated) and with  $^{35}$ S-labeled FlagI $\kappa$ B $\alpha$  for 60 min at 37°C in the presence of 3  $\mu$ M okadaic acid. Reaction products were subjected to 9% SDS-PAGE and analyzed by autoradiography. The doublet above the I $\kappa$ B $\alpha$  probably represents phosphorylation at one or both serines at positions 32 and 36.

(B) MEKK1 $\Delta$  and purified I $\kappa$ B $\alpha$  kinase, either alone or in combination, were incubated in the presence of [ $\gamma$ - $^{32}$ P]ATP. Reaction products were subjected to 8% SDS-PAGE and analyzed by autoradiography. Molecular weight markers (in kDa) are shown to the left. Dots indicate bands (approximately 200, 180, and 120 kDa) present when the I $\kappa$ B $\alpha$  kinase is incubated with [ $\gamma$ - $^{32}$ P]ATP in the absence of MEKK1 $\Delta$ . Bracket indicates bands present when MEKK1 $\Delta$  is incubated with [ $\gamma$ - $^{32}$ P]ATP in the absence of the I $\kappa$ B $\alpha$  kinase, showing MEKK1 $\Delta$  autophosphorylation.

Previous studies have implicated kinases other than MEKK1 in the activation of NF- $\kappa$ B. For example, PKA has been shown to dissociate the NF- $\kappa$ B-I $\kappa$ B complex (Ghosh and Baltimore, 1990), while PKC $\zeta$  coimmunoprecipitates with a factor that can phosphorylate I $\kappa$ B $\alpha$  (Diaz-Meco et al., 1994); with regard to the latter, it has been suggested that PKC $\zeta$  activates a kinase that phosphorylates I $\kappa$ B $\alpha$ . Neither PKA nor PKC $\zeta$ , however, phosphorylates I $\kappa$ B $\alpha$  at Ser-32 and -36, nor does either activate the I $\kappa$ B $\alpha$  kinase (Figure 6A). Additional kinases that have been implicated in NF- $\kappa$ B activation are raf-1 and the double-stranded RNA-activated protein kinase (PKR) (Finco and Baldwin, 1993; Yang et al., 1995). In preliminary experiments, we have been unable to observe activation of the I $\kappa$ B $\alpha$  kinase by enzymatically active c-raf (UBI) (data not shown). Additional studies will be required to determine the roles of raf and PKR in NF- $\kappa$ B regulation.

MEKK1 is a member of a family of enzymes that share a conserved C-terminal catalytic domain and may thus share overlap in substrates (Lange-Carter et al., 1993; Blank et al., 1996; Xu et al., 1996). Hence, it is conceivable that MEKK isoforms other than MEKK1 can activate the I $\kappa$ B $\alpha$  kinase. Different MEKK isoforms could potentially be involved in signaling responses to different stimuli. For example, MEKK1 has been shown to bind Ras

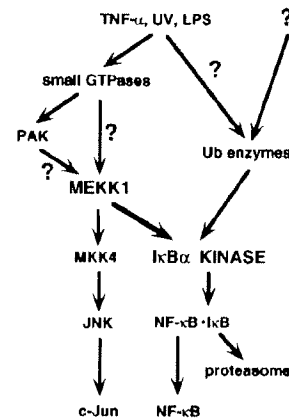


Figure 8. Model Showing Coordinate Activation of the I $\kappa$ B $\alpha$  Kinase and the JNK Pathway by MEKK1

Stressful stimuli such as TNF- $\alpha$ , UV irradiation, and lipopolysaccharide (LPS) activate MEKK1 by as-yet-to-be-identified mechanisms that involve small GTPases such as Rac1, Cdc42, and Ras and possibly the protein kinase PAK. MEKK1, in turn, coordinately activates the JNK pathway—which ultimately phosphorylates c-Jun—and the I $\kappa$ B $\alpha$  kinase. The I $\kappa$ B $\alpha$  kinase phosphorylates I $\kappa$ B $\alpha$  while still bound to NF- $\kappa$ B, targeting I $\kappa$ B $\alpha$  for degradation by the ubiquitin-proteasome pathway and thereby liberating NF- $\kappa$ B. The I $\kappa$ B $\alpha$  kinase can independently be activated by ubiquitination (Ub) enzymes (see Chen et al., 1996).

in a GTP-dependent manner and thus its activity may be regulated in a similar fashion (Russell et al., 1995). Indeed, Ha-Ras activation of NF- $\kappa$ B (Devary et al., 1993) could be mediated, in part, by activation of MEKK1. Aside from its interaction with Ras, little is known about the regulation and activation of MEKK1. The recent identification of MEKK1 as a large membrane-associated protein, with its C-terminal catalytic domain constituting less than 20% of the molecule, raises the possibility of complex modes of regulation (Xu et al., 1996). While yet to be demonstrated, other MEKKs could conceivably be regulated by other upstream regulators such as Rac1, Cdc42, and PAK. These data also leave open the possibility that there may be other I $\kappa$ B $\alpha$  kinases that respond to stimuli distinct from those that signal through MEKK1.

We as well as others (Hirano et al., 1996) have shown that dominant negative MEKK1 inhibits TNF- $\alpha$  activation of an NF- $\kappa$ B reporter gene in vivo. This is in contrast to a recent report that reveals no effect of dominant negative MEKK1 in similar experiments, which conclude that MEKK1 lies on a pathway distinct from that of the I $\kappa$ B $\alpha$  kinase (Liu et al., 1996). At present, we have no explanation for this discrepancy. However, our transfection results are strongly supported by the observation that the I $\kappa$ B $\alpha$  kinase is phosphorylated and activated by MEKK1 $\Delta$  in vitro. We thus conclude that the I $\kappa$ B $\alpha$  kinase and MEKK1, or minimally an MEKK isoform, are indeed part of the same pathway.

The I $\kappa$ B $\alpha$  kinase can be activated by ubiquitination independently of phosphorylation (Figure 8). To our knowledge, this dual regulation by phosphorylation or ubiquitination is unprecedented. Thus, the I $\kappa$ B $\alpha$  kinase itself can be considered a signal integrator, responding to both phosphorylation and ubiquitination. Different stimuli may therefore activate one, the other, or both

pathways. In principle, then, it may be possible to isolate an induced I $\kappa$ B $\alpha$  kinase species that is not ubiquitination-dependent, or one that is not phosphorylation-dependent. Indeed, the fractionation of highly purified I $\kappa$ B $\alpha$  kinase by ion exchange chromatography reveals kinase species that are phosphorylation but not ubiquitination inducible (Figures 5C and 5D).

A puzzling result from previous studies is that while I $\kappa$ B $\alpha$  kinase is easily assayed when present in HeLa cell S100 cytoplasmic extracts, the purified kinase is inactive, requiring ubiquitination components for activity (Chen et al., 1996). One possibility is that purification of the kinase separates the ubiquitination components from the kinase; hence, the purified kinase is inactive. The results described here raise a second and distinct possibility, namely that the I $\kappa$ B $\alpha$  kinase is activated by MEKK1 in the extract during the course of assay for I $\kappa$ B $\alpha$  kinase activity. Thus, purification of the I $\kappa$ B $\alpha$  kinase from S100 extracts removes it from both the ubiquitination components and MEKK1 present in the extract; in fact, Western blotting indicates that MEKK1 is not present in the I $\kappa$ B $\alpha$  kinase complex (data not shown). Either ubiquitination or MEKK1-dependent phosphorylation can activate the purified I $\kappa$ B $\alpha$  kinase (Figure 4C).

The detailed molecular mechanism by which MEKK1 activates the I $\kappa$ B $\alpha$  kinase remains to be determined. One possibility is that MEKK1 inactivates a negative regulatory subunit of the I $\kappa$ B $\alpha$  kinase, just as cAMP binds to and induces the dissociation of the regulatory subunit of PKA (Francis and Corbin, 1994). Alternatively, MEKK1 may activate the catalytic subunit of the I $\kappa$ B $\alpha$  kinase that subsequently phosphorylates Ser-32 and -36 of I $\kappa$ B $\alpha$ . Yet another possibility is that MEKK1 initiates a MAPK-like cascade within the I $\kappa$ B $\alpha$  kinase complex, with the terminal kinase the subunit that phosphorylates Ser-32 and -36; this would be somewhat analogous to the organization of MAPK modules as high molecular weight complexes in yeast (Choi et al., 1994). The incorporation of  $^{32}$ P into multiple subunits of the I $\kappa$ B $\alpha$  kinase complex in the presence of MEKK1 $\Delta$  (Figure 7) could be consistent with any of these possibilities. It will be of interest to determine if the I $\kappa$ B $\alpha$  kinase subunit(s) targeted by MEKK1 is the same as those targeted by the ubiquitination system. Further conclusions must await the identification and characterization of subunits of the I $\kappa$ B $\alpha$  kinase.

## Experimental Procedures

### Plasmids

pCMV5-MEKK1 (which encodes the C-terminal 672 residues of MEKK1), pcDNA3-FlagMEKK4, and pSR $\alpha$ HA-JNK1 were gifts of Dr. Roger Davis (University of Massachusetts, Worcester) and have been described (Derijard et al., 1994, 1995; Whitmarsh et al., 1995). pcDNA3-MEKK1 was constructed by subcloning the 2.4 kb EcoRI/EcoNI (blunt) fragment of pCMV5-MEKK1 encoding MEKK1 into the EcoRI/EcoRV site of pcDNA3. pcDNA3-FlagMEKK1 $\Delta$  (K432M) consists of an N-terminal Flag epitope fused to the C-terminal 321 amino acid fragment of MEKK1 with the indicated mutation (amino acid numbering according to Lange-Carter et al., 1993) and was constructed by polymerase chain reaction (Ausubel et al., 1989). pcDNA3-FlagMEKK1 $\Delta$  was constructed by replacing the 2.1 kb StuI fragment of pcDNA3-FlagMEKK1 $\Delta$  (K432M), which encodes the C-terminal 262 amino acids with the corresponding fragment of pcDNA3-MEKK1. pCMV4-FlagI $\kappa$ B $\alpha$  and pCMV4-FlagI $\kappa$ B $\alpha$ (S32A/

S36A) were gifts of Dr. Dean Ballard (Vanderbilt University) and have been described (Brockman et al., 1995). pcDNA1-cJun has been described (Du et al., 1993). pcDNA1-cJun(S63A/S73A) was constructed using overlapping polymerase chain reaction (Ausubel et al., 1989). pBS-I $\kappa$ B $\alpha$ , pBS-I $\kappa$ B $\alpha$ (S32A/S36A), pBS-FlagI $\kappa$ B $\alpha$ , pBS-FlagI $\kappa$ B $\alpha$ (S32A/S36A), (PRDII) $_2$ CAT, (PRDIV) $_2$ CAT, (CRE) $_2$ CAT, -110IFN- $\beta$ CAT, and pCMV-lacZ have been described (MacGregor and Caskey, 1989; Du and Maniatis, 1992; Thanos and Maniatis, 1992; Chen et al., 1995).

### Tissue Culture and Transfection

HeLa and L929 cells were maintained in DME media supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin. Transfections and virus infections, performed in 3.5 cm diameter wells, were conducted as described (Thanos and Maniatis, 1992). Cells were typically harvested at 41 to 49 hr posttransfection. CAT and  $\beta$ -galactosidase assays were performed as described (Sambrook et al., 1989). Protein concentrations were measured by the Bradford method.

### Immunoprecipitations

#### Transfected I $\kappa$ B $\alpha$

Cell pellets obtained by harvesting 3.5 cm diameter wells were lysed by the addition of 200  $\mu$ l of Buffer A (20 mM Tris, pH 7.5, 0.4 M KCl, 4 mM  $\beta$ -glycerolphosphate, 0.1 mM sodium orthovanadate, 0.1% NP-40, 10% glycerol, 10  $\mu$ g/ml leupeptin, 1 mM PMSF, and 1 mM DTT), followed by three freeze/thaw cycles. After centrifugation at 14,000  $\times$  g for 5 min at 4°C, the supernatant (320  $\mu$ g protein) was incubated with 20  $\mu$ l of M2-agarose (IBI-Kodak) in 1 ml of Buffer A with end-over-end rotation for 1 hr at 4°C. Resins were then washed three times with Buffer A and once with 0.1X Buffer A.

#### $^{35}$ S-Labeled FlagI $\kappa$ B $\alpha$

In vitro-translated FlagI $\kappa$ B $\alpha$  was immunoprecipitated by incubation with 10  $\mu$ l of M2-agarose in 1 ml of Buffer B (10 mM Tris, pH 7.6, 100 mM NaCl, 0.1% NP-40, 10  $\mu$ g/ml leupeptin, 1 mM DTT) with end-over-end rotation for 1 hr at 4°C. Resins were then washed three times with Buffer B, once with Buffer C (10 mM Tris, pH 7.6, 1 mg/ml BSA, 10  $\mu$ g/ml leupeptin, 1 mM DTT), and then eluted by the addition of 24  $\mu$ l of Buffer C containing 0.7 mg/ml Flag peptide for 30 min on ice.

### Western Blotting

Proteins were electrophoresed by SDS-PAGE and transferred to Immobilon-NC membranes (Millipore). The membranes were blocked with 5% nonfat milk and probed with rabbit anti-I $\kappa$ B $\alpha$  polyclonal antibodies (C21, Santa Cruz Biotechnology). Membranes were then incubated with goat anti-rabbit IgG-alkaline phosphatase or donkey anti-rabbit IgG-horseradish peroxidase conjugates, and developed using standard chromogenic or Enhanced Chemiluminescence (Amersham) substrates, respectively. Western blots of purified I $\kappa$ B $\alpha$  kinase employed antibodies (anti-MEKK1 [C22], anti-MKK4 [C20], anti-JNK1 [FL], anti-JNK2 [FL]) obtained from Santa Cruz Biotechnology.

### Extract Preparation

HeLa S $_3$  cell cytoplasmic extracts were prepared by two methods. In the first ("rapid lysis procedure"), mid-logarithmic growth phase HeLa S $_3$  cells cultured in RPMI 1640 media supplemented with 5% horse serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin were centrifuged at 2,600  $\times$  g for 10 min. Cells were resuspended in RPMI media containing 5% horse serum, and then either mock treated or incubated with 1000 U/ml TNF- $\alpha$  at 37°C. At various times, cells were centrifuged at 1,000  $\times$  g for 1 min at ambient temperature. Cells were then rapidly washed with ice-cold PBS, centrifuged again at 1,000  $\times$  g for 1 min, resuspended in ice-cold 50 mM Tris (pH 7.5), 1 mM EGTA, and then immediately lysed by dounce homogenization (15–20 strokes with an A-type pestle). The crude lysate was clarified by centrifugation at 4,600  $\times$  g for 10 min at 4°C, and the resulting supernatant immediately frozen at -80°C. In the second method ("S100"), HeLa S $_3$  cells were swollen in a hypotonic buffer and lysed, followed by removal of nuclei and centrifugation at 100,000  $\times$  g as described (Dignam et al., 1983). The supernatant was then dialyzed extensively against 20 mM Tris

(pH 7.5), 0.5 mM DTT. If not employed immediately, the extract was stored at  $-80^{\circ}\text{C}$ .

#### Purification of I $\kappa$ B $\alpha$ Kinase

HeLa cell S100 cytoplasmic extract, prepared as above, was applied to a Mono-Q anion exchange column. The I $\kappa$ B $\alpha$  kinase activity was eluted with 0.2–0.3 M KCl in Buffer D (50 mM Tris, pH 7.5, 0.5 mM DTT), and then precipitated with 40% ammonium sulfate. The resuspended precipitates were dialyzed against 10 mM  $\text{K}_2\text{HPO}_4$ - $\text{KH}_2\text{PO}_4$  (pH 7.0), 0.5 mM DTT, and then applied to a hydroxylapatite column. After elution with 0.2 M  $\text{K}_2\text{HPO}_4$ - $\text{KH}_2\text{PO}_4$  (pH 7.0), the kinase-containing fractions were applied to a Superdex-200 gel filtration column equilibrated with 50 mM Tris (pH 7.5), 0.5 mM DTT, and 150 mM NaCl. The high molecular weight fractions that contained the kinase activity were applied to a Mono-Q column and eluted with a linear gradient of 150–325 mM NaCl in Buffer D. Fractions from the Superdex-200 and second Mono-Q chromatographies were assayed for I $\kappa$ B $\alpha$  kinase activity in the presence of ubiquitination components (Ubc4 and ubiquitin, in addition to E1 supplied by the wheat germ extract employed for in vitro translation of I $\kappa$ B $\alpha$ ) (Chen et al., 1996), or recombinant MEKK1 $\Delta$ .

#### Preparation of Recombinant Proteins

(His) $_6$ MEKK1 $\Delta$  and (His) $_6$ MEKK1 $\Delta$  (K432M) were purified using Ni-NTA agarose from Sf9 cells infected with baculovirus prepared using the Bac-to-Bac Expression System (GIBCO-BRL Life Technologies). pFastBacHT-MEKK1 $\Delta$  and pFastBacHT-MEKK1 $\Delta$  (K432M) were constructed by subcloning the 1.2 kb NcoI/XbaI coding sequence fragment of pcDNA3-FlagMEKK1 $\Delta$  and pcDNA3-FlagMEKK1 $\Delta$  (K432M), respectively, into the NcoI/XbaI site of pFastBacHT $\Delta$ . Recombinant bacmids and baculovirus were subsequently prepared according to the manufacturer's instructions. GST-MKK4 and GST-JNK1 were purified from *E. coli* HB101 transformed with pGEX-MKK4 and pGEX-JNK1, respectively, employing glutathione agarose affinity chromatography as described (Smith and Johnson, 1988). pGEX-MKK4 was constructed by subcloning the 1.1 kb BamHI (blunt)/Bsp120I (blunt) fragment of pcDNA3-FlagMKK4 containing the MKK4 coding sequence into the SmaI site of pGEX-3X (Pharmacia). pGEX-JNK1 was constructed by subcloning the 1.4 kb NcoI (blunt)/SalI fragment of pSR $\alpha$ HA-JNK1 containing the JNK1 coding sequence into the EcoRI (blunt)/SalI site of pGEX-5X-1 (Pharmacia). (His) $_6$ I $\kappa$ B $\alpha$  was purified using Ni-NTA agarose from *E. coli* BL21(DE3)lysS transformed with pRSET-I $\kappa$ B $\alpha$ . pRSET-I $\kappa$ B $\alpha$  was constructed by subcloning the EagI (blunt)/HindIII fragment of pBS-I $\kappa$ B $\alpha$  containing the I $\kappa$ B $\alpha$  coding sequence into the PvuII/HindIII site of pRSET A (Invitrogen). The E2 enzymes Ubc4 and GST-Ubc5 were prepared as described (Chen et al., 1996). Concentrations of recombinant proteins were determined by SDS-PAGE followed by staining with Coomassie blue and comparison with bovine serum albumin standards.

#### Protein Kinase Assays

##### Mobility Shift Assays

Typically, HeLa cell cytoplasmic extracts or purified I $\kappa$ B $\alpha$  kinase (from gel filtration chromatography as described above) was incubated with 0.5  $\mu\text{l}$  of in vitro-translated,  $^{35}\text{S}$ -labeled protein in a total volume of 10  $\mu\text{l}$  containing 50 mM Tris (pH 7.6), 5 mM  $\text{MgCl}_2$ , 2 mM ATP, 10 mM phosphocreatine, 3.5 U/ml creatine phosphokinase, and 2.5  $\mu\text{M}$  okadaic acid. In vitro-translated,  $^{35}\text{S}$ -labeled I $\kappa$ B $\alpha$ , Flag-I $\kappa$ B $\alpha$ , and c-Jun, or their phosphorylation defective mutants, were prepared using TnT wheat germ extract kits (Promega) and pBS-I $\kappa$ B $\alpha$ , pBS-I $\kappa$ B $\alpha$  (S32A/S36A), pBS-FlagI $\kappa$ B $\alpha$ , pBS-FlagI $\kappa$ B $\alpha$  (S32A/S36A), pcDNA1-cJun, or pcDNA1-cJun(S63A/S73A) as templates.

##### $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ Labeling of I $\kappa$ B $\alpha$

Enzyme was incubated with 0.5  $\mu\text{g}$  (His) $_6$ I $\kappa$ B $\alpha$  in 10  $\mu\text{l}$  of 50 mM Tris (pH 7.6), 5 mM  $\text{MgCl}_2$ , 2.5  $\mu\text{M}$  okadaic acid, 200  $\mu\text{M}$  ATP and 5  $\mu\text{Ci}$  of  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ . Incubations were carried out at  $30^{\circ}\text{C}$  for 30 min.

#### Dephosphorylation of I $\kappa$ B $\alpha$ Kinase Complex

Purified I $\kappa$ B $\alpha$  kinase (from gel filtration chromatography) was treated with MEKK1 $\Delta$  in 50 mM Tris (pH 7.6), 5 mM  $\text{MgCl}_2$ , 2 mM ATP for 30 min at  $30^{\circ}\text{C}$ . MEKK1 $\Delta$ -activated I $\kappa$ B $\alpha$  kinase was separated from ATP by centrifugal gel filtration on Sephadex G50 and subsequently

incubated with or without calf intestinal alkaline phosphatase (CIP) in 50 mM Tris (pH 7.8), 0.1 mM EDTA for 30 min at  $30^{\circ}\text{C}$ . I $\kappa$ B $\alpha$  kinase was then separated from CIP and MEKK1 $\Delta$  by chromatography on a Superdex 200 column and assayed for I $\kappa$ B $\alpha$  kinase activity in the absence or presence of MEKK1 $\Delta$ .

#### $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ Labeling of I $\kappa$ B $\alpha$ Kinase Complex

Two nanograms of MEKK1 $\Delta$  was incubated in 7  $\mu\text{l}$  of 70 mM Tris (pH 7.6), 7 mM  $\text{MgCl}_2$ , 3.5  $\mu\text{M}$  okadaic acid, and 140  $\mu\text{M}$  ATP for 15 min at  $30^{\circ}\text{C}$ . Subsequently, purified I $\kappa$ B $\alpha$  kinase (from the second Mono Q chromatography step as described above) and 10  $\mu\text{Ci}$  of  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  in a total volume of 3  $\mu\text{l}$  were added and the incubation continued at  $30^{\circ}\text{C}$  for an additional 30 min. In control reactions, either MEKK1 $\Delta$  or I $\kappa$ B $\alpha$  kinase was omitted.

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